

Feedback-Resistant Mevalonate Kinases

The present invention provides modified mevalonate kinases that are less sensitive to feedback inhibition. The modified enzymes and polynucleotides encoding the same can be used for the production of isoprenoid compounds, for the treatment of disorders that are characterized by decreased mevalonate kinase activity, and for diagnostic purposes.

Mevalonate kinase (MK) is an essential enzyme in the mevalonate pathway which leads to the production of numerous cellular isoprenoids. Isopentenyl diphosphate (IPP), the product of the mevalonate pathway, and the isomeric compound, dimethylallyl diphosphate (DMAPP), are the fundamental building blocks of isoprenoids in all organisms. The isoprenoids include more than 23,000 naturally occurring molecules of both primary and secondary metabolism. The chemical diversity of this natural product class reflects their wide-ranging physiological roles in all living systems. Isoprenoids include, e.g., hopane triterpenes, ubiquinones and menaquinones in bacteria, carotenoids, plastoquinones, mono-, sesqui-, di-, and tri-terpenes, and the prenyl side chains of chlorophylls in plants, and heme A, quinones, dolichols, sterols/steroids and retinoids in mammals. In addition, isoprenoids are involved in isopentenyl tRNAs, in protein prenylation and in cholesterol modification of, e.g., the hedgehog class of cell signaling proteins.

The MK enzyme has been characterized both at the biochemical and the molecular level in a variety of organisms (Houten et al., Biochim. Biophys. Acta 1529, 19-32, 2000). Already now, the DNA and amino acid sequences of many mevalonate kinases are known (e.g., Swiss-Prot accession numbers/IDs P07277/kime_yeast; Q9R008/kime_mouse; P17256/kime_rat; Q03426/kime_human; P46086/kime_arath; Q09780/kime_schpo; Q9V187/kime_pyra; O59291/kime_pyrho; Q8U0F3/kime_pyrfu; Q50559/kime_metth; O27995/kime_arclu; Q58487/kime_metja; Q9Y946/kime_aerpe), and every month, new entries can be added to the list of known mevalonate kinase sequences. The above sequences which have been obtained from genome sequencing projects have been assigned putative mevalonate kinase function based on sequence similarity with known mevalonate kinases. However, for those skilled in the art, it is straightforward to prove that these sequences in fact code for proteins with mevalonate kinase activity.

In terms of regulation, HMG-CoA reductase is considered broadly to be the rate-determining enzyme in the mevalonate pathway (e.g., Goldstein and Brown, Nature 343, 425-430, 1990; Weinberger, Trends Endocrinol. Metab. 7, 1-6, 1996; Hampton et al., Trends Biochem. Sci. 21, 140-145, 1996; Houten et al., J. Biol. Chem. 278, 5736-5743, 2003). In line with this view, supplementation of the culture medium with mevalonate has been shown to stimulate carotenoid production in both *Phaffia rhodozyma* (Calo et al., Biotechnol. Lett. 17, 575-578, 1995) and *Haematococcus pluvialis* (Kobayashi et al., J. Ferment. Bioeng. 71, 335-339, 1991). Increasing evidence in recent years, however, indicates that mevalonate kinase is subject to feedback inhibition by, e.g., the down-stream products geranyldiphosphate, farnesyl diphosphate and geranylgeranyldiphosphate. This feedback inhibition may also contribute to regulation and rate limitation of the mevalonate pathway and, thus, of isoprenoid biosynthesis in general.

In humans, the importance of mevalonate kinase was demonstrated by the identification of its deficiency as the biochemical and molecular cause of the inherited human disorders mevalonic aciduria and hyperimmunoglobulinemia D and periodic fever syndrome (Houten et al., 2000; Nwokoro et al., Mol. Genet. Metab. 74, 105-119, 2001). The pathophysiology of these disorders is not yet understood, but eventually will give insight into the in vivo role of mevalonate kinase and isoprenoid biosynthesis with respect to the acute phase response and fever. Mevalonate kinase deficiency also seems to be involved, e.g., in Zellweger syndrome and in rhizomelic chondrodysplasia punctata, a disorder of peroxisomal biogenesis wherein a subset of peroxisomal enzymes, including mevalonate kinase, is not transported into peroxisomes (Kelley and Herman, Annu. Rev. Genomics Hum. Genet. 2, 299-341, 2001). Finally, mevalonate kinase was proposed to play a role in cellular proliferation, cell cycle regulation and/or cellular transformation (see Graef et al., Virology 208, 696-703, 1995; Hinson et al., J. Biol. Chem. 272, 26756-26760, 1997).

All mevalonate kinases investigated so far are feedback-inhibited by downstream products of the pathway. No mevalonate kinase has so far been described to be resistant to feedback inhibition by, e.g., farnesyl pyrophosphate or geranylgeranyl pyrophosphate. Feedback-resistant mevalonate kinase enzymes may have industrial potential, e.g., (1) in the biotechnological production of all kinds of isoprenoid compounds (e.g., carotenoids, coenzyme Q10, vitamin D, sterols, etc.), (2) as diagnostic enzymes for, e.g., enzymatic measurement of mevalonate concentrations in biological fluids, or (3) as therapeutic enzymes for lowering mevalonate concentrations in patients with mevalonic aciduria. Feedback-resistant MKs are particularly suited for biotechnological production of isoprenoids, since they may allow a larger flux through the mevalonate pathway and, thus, higher isoprenoid productivity.

As used herein, the term "mevalonate kinase" shall mean any enzyme that is capable of catalyzing the phosphorylation of mevalonate (mevalonic acid) to 5-phosphomevalonate (5-phosphomevalonic acid), or of mevalonate analogues (as, e.g., described by Wilde and Eggerer, Eur. J. Biochem. 221, 463-473, 1994) to the corresponding phosphorylated compounds. To afford phosphorylation of mevalonate (or mevalonate analogues), the enzyme requires additionally a suitable phosphate donor. As phosphate donors for mevalonate kinase, different compounds are conceivable. The most preferred phosphate donor is ATP (adenosine 5'-triphosphate). Other preferred phosphate donors are TTP, ITP, GTP, UTP, or CTP (see Gibson et al., Enzyme 41, 47-55, 1989). A "mevalonate kinase" may be homologous to one or more of the enzymes the amino acid sequences of which are shown in SEQ ID NOs:1 to 14. "Homologous" refers to a mevalonate kinase that is at least about 60% identical, preferably at least about 70% identical, more preferably at least about 80% identical, even more preferably at least about 90% identical, most preferably at least about 95% identical to one or more of the amino acid sequences as shown in SEQ ID NOs:1 to 14 and 30.

The term "% identity", as known in the art, means the degree of relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" can be readily determined by known methods, e.g., with the program GAP (GCG Wisconsin Package, version 10.2, Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752, USA) using the following parameters: gap creation penalty 8, gap extension penalty 2 (default parameters).

"Wild-type enzyme" or "wild-type mevalonate kinase" shall mean any mevalonate kinase homologous to any one of SEQ ID Nos:1-14 and 30 that is used as starting point for designing (more) feedback resistant mutants according to the present invention. Inherently, this definition implies that such a "wild-type enzyme" or "wild-type mevalonate kinase" is sensitive to inhibition to physiologically or industrially relevant concentrations of a downstream product of the mevalonate pathway, e.g., FPP or GGPP. "Wild-type" in the context of the present invention shall not restrict the scope of the invention to only mevalonate kinases/mevalonate kinase sequences only derivable from nature. It shall be explicitly stated here that also variants of synthetic mevalonate kinases (as long as they are homologous to any one of SEQ ID Nos:1-14 and 30) are termed "wild-type", if they can be made (more) feedback resistant by any of the teachings of the present invention. The terms "wild-type mevalonate kinase" and "non-modified mevalonate kinase" are used interchangeably herein.

A "mutant", "mutant enzyme", or "mutant mevalonate kinase" shall mean any variant derivable from a given wild-type enzyme/mevalonate kinase (according to the above definition) according to the teachings of the present invention and being (more) feedback

resistant than the respective wild-type enzyme. For the scope of the present invention, it is not relevant how the mutant(s) are obtained; such mutants can be obtained, e.g., by site-directed mutagenesis, saturation mutagenesis, random mutagenesis/directed evolution, chemical or UV mutagenesis of entire cells/organisms, etc. These mutants can also be prepared, e.g., by designing synthetic genes, and/or by in vitro (cell-free) translation (see, 5 e.g., Jermutus et al., Curr. Opin. Biotechnol. 9, 534-548, 1998; Betton, Curr. Prot. Pept. Sci. 4, 73-80, 2003; Martin et al., Biotechniques 31, 948-, 2001). For testing of feedback resistance, mutants can be generated by methods known to those skilled in the art (e.g., by site-directed mutagenesis or by designing synthetic genes).

10 "Isoprenoid" in the context of this patent application shall include any and all metabolite(s) and prenylated macromolecule(s) derivable from mevalonate by either natural or non-natural pathways (i.e., pathways not occurring in nature, but engineered biotechnologically), preferably biochemical pathways. Isoprenoids include but are not limited to hopane triterpenes, ubiquinones and menaquinones in bacteria, carotenoids, 15 plastoquinones, mono-, sesqui-, di-, and tri-terpenes, and the prenyl side chains of chlorophylls in plants, and heme A, quinones, coenzyme Q10, dolichols, sterols/steroids, vitamin D, retinoids, and the like.

It is in general an object of the present invention to provide a mevalonate kinase which has been modified in a way that its catalytic properties are more favorable (i.e., less sensitive to 20 feedback inhibition) than those of the non-modified mevalonate kinase.

The invention relates to a modified mevalonate kinase which exhibits a sensitivity to feedback inhibition which is reduced in comparison to the corresponding non-modified mevalonate kinase wherein

25 (i) the amino acid sequence of the modified mevalonate kinase contains at least one mutation when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase and

(ii) the at least one mutation is at one or more amino acid positions selected from the group consisting of amino acid positions corresponding to positions 17, 47, 93, 94, 132, 167, 169, 204, and 266 of the amino acid sequence of *Paracoccus zeaxanthinifaciens* 30 mevalonate kinase as shown in SEQ ID NO:1.

As used herein, the term "feedback inhibition" denotes the inhibition of enzymatic activity of mevalonate kinase by a metabolite downstream of mevalonate in isoprenoid biosynthesis. Metabolites downstream of mevalonate in isoprenoid biosynthesis include but are not limited to 5-phosphomevalonate, isopentenyl diphosphate (IPP), 3,3- 35 dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl diphosphate

(FPP), geranylgeranyl diphosphate (GGPP), farnesol, dolichol phosphate, and phytyl-pyrophosphate (Dorsey and Porter, J. Biol. Chem. 243, 4667-4670, 1968; Flint, Biochem. J. 120, 145-150, 1970; Gray and Kekwick, Biochim. Biophys. Acta 279, 290-296, 1972; Hinson et al., J. Lipid Res. 38, 2216-2223, 1997). It is believed that feedback inhibition of mevalonate kinase is based on allosteric regulation of mevalonate kinase by binding to the enzyme of the metabolite downstream of mevalonate in isoprenoid biosynthesis.

Preferably, the feedback inhibition is feedback inhibition by farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP).

According to the present invention the modified mevalonate kinase exhibits a sensitivity to feedback inhibition which is reduced in comparison to the corresponding non-modified mevalonate kinase. Preferably, the sensitivity to feedback inhibition of the modified mevalonate kinase of the invention is reduced by at least 5% in comparison to the corresponding non-modified mevalonate kinase (for measurement and quantification of feedback resistance, see below).

"Feedback resistance" shall mean any increase in resistance to "feedback inhibition" (as defined above). Feedback resistance can be analyzed in different ways known to those skilled in the art. An appropriate approach shall be described here shortly: mevalonate kinase activity is measured in an activity assay similar to the one described in Example 2 at non-saturating concentrations of ATP (or of another phosphate donor) and mevalonate (or mevalonate analogue), *i.e.*, at ATP (or phosphate donor) and mevalonate (or mevalonate analogue) concentrations around which the reaction rate is sensitive to changes of these substrate concentrations, *e.g.*, at concentrations around the respective K_m values of the enzyme under investigation for these substrates. The activities of both wild-type mevalonate kinase and of a variant/mutant of this enzyme are measured under otherwise identical conditions both in the absence and presence of a relevant concentration of a feedback inhibitor, *i.e.*, at a concentration of feedback inhibitor affording significant inhibition of the wild-type mevalonate kinase. If the extent of inhibition (*e.g.*, % inhibition) by the feedback inhibitor is lower for the mutant than for the wild-type enzyme, then the mutant is "feedback resistant" in the context of the present patent application. Once a "feedback resistant" variant/mutant has been identified, the same procedure as described above can be applied to identify further improved mutants, *i.e.*, mutants that are even more feedback resistant. Feedback resistance (%) is calculated as follows: if a and b are the measured mevalonate kinase activities of the wild-type enzyme in the absence and presence, respectively, of the feedback inhibitor (*e.g.*, FPP), and if c and d are the measured mevalonate kinase activities of the mutant enzyme in the absence and presence, respectively, of the same feedback inhibitor, then % feedback resistance is:

$$\% \text{ resistance} = 100 \cdot ((d/c) - (b/a)) / (1 - (b/a))$$

Preferably, the feedback resistance refers to the experimental conditions described in Example 2 of this application. Approximately 3-30 mU/ml (corresponding to approx. 1-10 µg/ml of *Paracoccus zeaxanthinifaciens* mevalonate kinase), preferably approx. 10-20 mU/ml of mevalonate kinase activity, and optionally 46 µM FPP were present in the assay mixture, and the reaction was carried out at 30°C.

The modified mevalonate kinase of the invention exhibits a feedback resistance of at least 5%, preferably at least about 10%, more preferably at least about 25%, even more preferably at least about 40%, still more preferably at least about 60%, most preferably at least about 70% when compared with the corresponding non-modified mevalonate kinase.

The amino acid sequence of the modified mevalonate kinase of the invention contains at least one mutation when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase. The mutation may be an addition, deletion and/or substitution. Preferably, the mutation is an amino acid substitution wherein a given amino acid present in the amino acid sequence of the non-modified mevalonate kinase is replaced with a different amino acid in the amino acid sequence of the modified mevalonate kinase of the invention. The amino acid sequence of the modified mevalonate kinase may contain at least one amino acid substitution when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase. In further embodiments, the modified mevalonate kinase contains at least two, at least three, at least four or at least five substitutions when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase. In other embodiments of the invention, the modified mevalonate kinase contains one to ten, one to seven, one to five, one to four, two to ten, two to seven, two to five, two to four, three to ten, three to seven, three to five or three to four amino acid substitutions when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase.

The one or more mutation(s) may be at one or more amino acid position(s) selected from the group consisting of amino acid positions corresponding to positions 17, 47, 93, 94, 132, 167, 169, 204, and 266 of the amino acid sequence of *Paracoccus zeaxanthinifaciens* mevalonate kinase as shown in SEQ ID NO:1.

Preferably, the at least one mutation is at one or more amino acid positions selected from the group consisting of amino acid positions corresponding to positions 17, 47, 93, 94, 132, 167, and 266 of the amino acid sequence of *Paracoccus zeaxanthinifaciens* mevalonate kinase as shown in SEQ ID NO:1. In another preferred embodiment the at least one mutation is at one or more amino acid positions selected from the group consisting of

amino acid positions corresponding to positions 17, 47, 93, 94, 132, 167, and 169 of the amino acid sequence of *Paracoccus zeaxanthinifaciens* mevalonate kinase as shown in SEQ ID NO:1.

5 If the modified mevalonate kinase contains only a single amino acid substitution when compared to the corresponding non-modified mevalonate kinase it is preferred that the single amino acid substitution is at a position selected from the group consisting of positions corresponding to the amino acid positions 17, 47, 93, 94, 204 and 266 of SEQ ID NO:1. More preferably, the substitution is I17T, G47D, K93E, V94I, R204H or C266S.

10 In a particularly preferred embodiment, the mutation is a substitution which affects the amino acid position corresponding to amino acid position 17 of the amino acid sequence as shown in SEQ ID NO:1. The amino acid present in the non-modified mevalonate kinase is preferably isoleucine. The amino acid in the sequence of the non-modified mevalonate kinase may be changed to either threonine or alanine. Most preferably, the substitution at the amino acid position corresponding to position 17 of the sequence as
15 shown in SEQ ID NO:1 consists of the replacement of isoleucine with threonine.

If the modified mevalonate kinase contains at least two mutations when compared to the corresponding non-modified mevalonate kinase, one of the mutations may be at the amino acid position corresponding to position 375 of SEQ ID NO:1. If the modified mevalonate kinase contains two amino acid substitutions when compared to the corresponding non-
20 modified mevalonate kinase it is preferred that the amino acid substitutions are at positions corresponding to combinations of positions 132/375, 167/169, 17/47 or 17/93 of SEQ ID NO:1. Most preferred are the combinations P132A/P375R, R167W/K169Q, I17T/G47D or I17T/K93E.

25 If the modified mevalonate kinase contains three amino acid substitutions when compared to the corresponding non-modified mevalonate kinase it is preferred that the amino acid substitutions are at positions corresponding to combinations of positions 17/167/169, 17/132/375, 93/132/375, or 17/47/93 of SEQ ID NO:1. Most preferred are the combinations I17T/R167W/K169Q, I17T/P132A/P375R, K93E/P132A/P375R, I17T/R167W/K169H, I17T/R167T/K169M, I17T/R167T/K169Y, I17T/R167F/K169Q,
30 I17T/R167I/K169N, I17T/R167H/K169Y, I17T/G47D/K93E or I17T/G47D/K93Q.

If the modified mevalonate kinase contains four amino acid substitutions when compared to the corresponding non-modified mevalonate kinase it is preferred that the amino acid substitutions are at positions corresponding to combinations of positions 17/47/93/132 of
35 SEQ ID NO:1. Most preferred are the combinations I17T/G47D/K93E/P132A or I17T/G47D/K93E/P132S.

Most preferred are the combinations of mutations disclosed in Table 1, 2, 3 or 4 (see infra). The amino acid positions identified in these examples may be transferred to mevalonate kinases of different origin.

5 The modified mevalonate kinase of the invention may be obtained by introducing a mutation to the corresponding non-modified mevalonate kinase. A non-modified mevalonate kinase may be any mevalonate kinase which exhibits sensitivity to feedback inhibition. Non-modified mevalonate kinases include but are not limited to mevalonate kinases derivable from nature. Non-modified mevalonate kinases further include
10 shown in SEQ ID NOs:1 to 14 and 30.

Preferred non-modified mevalonate kinases include those having a sequence selected from the group consisting of the amino acid sequences as shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID
15 NO:14 and SEQ ID NO:30.

The non-modified mevalonate kinase may be of eukaryotic or prokaryotic, preferably fungal or bacterial origin, more preferably *Aspergillus* or *Saccharomyces* or *Paracoccus* or *Phaffia* and most preferably *Aspergillus niger* or *Saccharomyces cerevisiae* or *Paracoccus zeaxanthinifaciens* or *Phaffia rhodozyma*, origin. In one embodiment, the non-modified
20 mevalonate kinase is of prokaryotic, preferably bacterial origin, more preferably *Paracoccus* and most preferably *Paracoccus zeaxanthinifaciens* origin.

Preferably, the feedback inhibition of the non-modified mevalonate kinase by FPP is at least 10%, more preferably at least 20%, still more preferably at least 30%, even more preferably at least 40%, most preferably at least 50% as determined in an assay described in
25 Example 2 (0 or 46 μ M FPP).

The modified mevalonate kinase of the invention may comprise foreign amino acids, preferably at its N- or C-terminus. "Foreign amino acids" mean amino acids which are not present in a native (occurring in nature) mevalonate kinase, preferably a stretch of at least about 3, at least about 5 or at least about 7 contiguous amino acids which are not present
30 in a native mevalonate kinase. Preferred stretches of foreign amino acids include but are not limited to "tags" that facilitate purification of the recombinantly produced modified mevalonate kinase. Examples of such tags include but are not limited to a "His₆" tag, a FLAG tag, a myc tag, and the like.

In another embodiment the modified mevalonate kinase may contain one or more, e.g.
35 two, deletions when compared with the amino acid sequence of the corresponding non-

modified mevalonate kinase. Preferably, the deletions affect N- or C-terminal amino acids of the corresponding non-modified mevalonate kinase and do not significantly reduce the functional properties, *e.g.*, the specific activity, of the enzyme.

5 The modified mevalonate kinase of the invention usually is a non-naturally occurring mevalonate kinase. Preferably, the specific activity of the modified mevalonate kinase is at least 10%, more preferably at least 20%, still more preferably at least 35%, even more preferably at least 50%, most preferably at least 75% of the specific activity of the corresponding non-modified mevalonate kinase.

10 The modified mevalonate kinase of the invention may be an isolated polypeptide. As used herein, the term "isolated polypeptide" refers to a polypeptide that is substantially free of other polypeptides. An isolated polypeptide is preferably greater than 80% pure, preferably greater than 90% pure, more preferably greater than 95% pure, most preferably greater than 99% pure. Purity may be determined according to methods known in the art, *e.g.*, by SDS-PAGE and subsequent protein staining. Protein bands can then be quantified by
15 densitometry. Further methods for determining the purity are within the level of ordinary skill.

The invention further relates to a polynucleotide comprising a nucleotide sequence which codes for a modified mevalonate kinase according to the invention. "Polynucleotide" as used herein refers to a polyribonucleotide or polydeoxyribonucleotide that may be
20 unmodified RNA or DNA or modified RNA or DNA. Polynucleotides include but are not limited to single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-
25 stranded regions. The term "polynucleotide" includes DNA or RNA that comprises one or more unusual bases, *e.g.*, inosine, or one or more modified bases, *e.g.*, tritylated bases.

The polynucleotide of the invention can easily be obtained by modifying a polynucleotide sequence which codes for a non-modified mevalonate kinase. Examples of such polynucleotide sequences encoding non-modified mevalonate kinases are shown in SEQ
30 ID NOs:16 to 29 and 31. Methods for introducing mutations, *e.g.*, additions, deletions and/or substitutions into the nucleotide sequence coding for the non-modified mevalonate kinase include but are not limited to site-directed mutagenesis and PCR-based methods.

The principles of the polymerase chain reaction (PCR) method are outlined, *e.g.*, by White *et al.*, Trends Genet. 5, 185-189, 1989, whereas improved methods are described, *e.g.*, in

Innis et al. [PCR Protocols: A guide to Methods and Applications, Academic Press, Inc. (1990)].

DNA sequences of the present invention can be constructed starting from genomic or cDNA sequences coding for mevalonate kinases known in the state of the art [for sequence information see, e.g., the relevant sequence databases, for example Genbank
5 (Intelligenetics, California, USA), European Bioinformatics Institute (Hinston Hall, Cambridge, GB), NBRF (Georgetown University, Medical Centre, Washington DC, USA) and Vecbase (University of Wisconsin, Biotechnology Centre, Madison, Wisconsin, USA) or the sequence information disclosed in the figures and sequence listing] by methods of *in vitro* mutagenesis [see e.g. Sambrook et al., Molecular Cloning, Cold Spring Harbor
10 Laboratory Press, New York]. A widely used strategy for such "site directed mutagenesis", as originally outlined by Hutchison and Edgell (J. Virol. 8, 181-189, 1971), involves the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution to a target region of a single-stranded DNA sequence wherein the mutation should be
15 introduced (for review see Smith, Annu. Rev. Genet. 19, 423-462, 1985; and for improved methods see references 2-6 in Stanssen et al., Nucl. Acids Res. 17, 4441-4454, 1989). Another possibility of mutating a given DNA sequence which is also preferred for the practice of the present invention is mutagenesis by using the polymerase chain reaction (PCR). DNA as starting material can be isolated by methods known in the art and
20 described, e.g., in Sambrook et al. (Molecular Cloning) from the respective strains/organisms. It is, however, understood that DNA encoding a mevalonate kinase to be constructed/mutated in accordance with the present invention can also be prepared on the basis of a known DNA sequence, e.g. by construction of a synthetic gene by methods known in the art (as described, e.g., in EP 747 483 and by Lehmann et al., Prot. Eng. 13, 49-
25 57, 2000).

Non-limiting examples of polynucleotides encoding modified mevalonate kinases according to the invention are shown in SEQ ID NO: 32 and 33.

The polynucleotide of the invention may be an isolated polynucleotide. The term "isolated polynucleotide" denotes a polynucleotide that is substantially free from other nucleic acid
30 sequences such as but not limited to other chromosomal and extrachromosomal DNA and RNA. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also embraces recombinant polynucleotides and chemically synthesized polynucleotides.

In yet another embodiment the invention pertains to a vector or plasmid comprising a
35 polynucleotide according to the invention. The vector or plasmid preferably comprises at least one marker gene. The vector or plasmid may further comprise regulatory elements

operably linked to the polynucleotide of the invention. The term "operably linked" as used herein refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence, *i.e.*, the coding sequence is under the transcriptional control of the promoter. Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation. The term "expression" denotes the transcription of a DNA sequence into mRNA and/or the translation of mRNA into an amino acid sequence. The term "overexpression" means the production of a gene product in a modified organism (*e.g.*, modified by transformation or transfection) that exceeds levels of production in the corresponding non-modified organism.

Once complete DNA sequences of the present invention have been obtained they can be integrated into vectors by methods known in the art and described in, *e.g.*, Sambrook et al. (s.a.) to (over-) express the encoded polypeptide in appropriate host systems. However, a man skilled in the art knows that also the DNA sequences themselves can be used to transform the suitable host systems of the invention to get (over-) expression of the encoded polypeptide. Appropriate host systems are for example fungi, like *Aspergilli*, *e.g.* *Aspergillus niger* or *Aspergillus oryzae*, or like *Trichoderma*, *e.g.* *Trichoderma reesei*, or yeasts like *Saccharomyces*, *e.g.* *Saccharomyces cerevisiae*, or *Pichia*, like *Pichia pastoris*, or *Hansenula polymorpha*, *e.g.* *H. polymorpha* (DSM5215). A man skilled in the art knows that such microorganisms are available from depository authorities, *e.g.* the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS) or the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ) or any other depository authority as listed in the Journal "Industrial Property" (vol. 1, pages 29-40, 1991) or in the Official Journal of the European Patent Office (vol. 4, pages 155/156, 2003). Bacteria which can be used are, *e.g.*, *Paracoccus*, as *e.g.* *Paracoccus zeaxanthinifaciens*, *E. coli*, *Bacilli* as, *e.g.*, *Bacillus subtilis* or *Streptomyces*, *e.g.* *Streptomyces lividans* (see *e.g.* Anné and van Mellaert in FEMS Microbiol. Lett. 114, 121-128, 1993. *E. coli* which could be used are, *e.g.*, *E. coli* K12 strains, *e.g.* M15 (described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474, 1974), HB 101 (ATCC No. 33694) or *E. coli* SG13009 (Gottesman et al., J. Bacteriol. 148, 265-273, 1981).

Vectors which can be used for expression in fungi are known in the art and described *e.g.* in EP 420 358, or by Cullen et al. (Bio/Technology 5, 369-376, 1987), Ward (in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York, 1991), Upshall et al. (Bio/Technology 5, 1301-1304, 1987), Gwynne et al. (Bio/Technology 5, 71-79, 1987), or Punt et al. (J. Biotechnol. 17, 19-34, 1991), and for yeast by Sreekrishna et al. (J. Basic Microbiol. 28, 265-278, 1988; Biochemistry 28, 4117-

4125, 1989), Hitzemann et al. (Nature 293, 717-722, 1981) or in EP 183 070, EP 183 071, EP 248 227, EP 263 311. Suitable vectors which can be used for expression in *E. coli* are mentioned, e.g., by Sambrook et al. [s.a.] or by Fiers et al. in Proc. 8th Int. Biotechnol. Symp. [Soc. Franc. de Microbiol., Paris (Durand et al., eds.), pp. 680-697, 1988], Bujard et al. (in Meth. Enzymol., eds. Wu and Grossmann, Academic Press, Inc., Vol. 155, 416-433, 1987), or Stüber et al. (in Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152, 1990). Vectors which could be used for expression in Bacilli are known in the art and described, e.g. in EP 207 459 or EP 405 370, by Yansura and Henner in Proc. Natl. Acad. Sci. USA 81, 439-443 (1984), or by Henner, Le Grice and Nagarajan in Meth. Enzymol. 185, 199-228, 1990. Vectors which can be used for expression in *H. polymorpha* are known in the art and described, e.g., in Gellissen et al., Biotechnology 9, 291-295, 1991.

Either such vectors already carry regulatory elements, e.g. promoters, or the DNA sequences of the present invention can be engineered to contain such elements. Suitable promoter elements which can be used are known in the art and are, e.g., for *Trichoderma reesei* the cbh1- (Haarki et al., Biotechnology 7, 596-600, 1989) or the pki1-promoter (Schindler et al., Gene 130, 271-275, 1993), for *Aspergillus oryzae* the amy-promoter [Christensen et al., Abstr. 19th Lunteren Lectures on Molecular Genetics F23 (1987); Christensen et al., Biotechnology 6, 1419-1422, 1988; Tada et al., Mol. Gen. Genet. 229, 301-306, 1991], for *Aspergillus niger* the glaA- (Cullen et al., Bio/Technology 5, 369-376, 1987; Gwynne et al., Bio/Technology 5, 713-719, 1987; Ward in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York, 83-106, 1991), alcA- (Gwynne et al., Bio/Technology 5, 718-719, 1987), suc1- (Boddy et al., Curr. Genet. 24, 60-66, 1993), aphA- (MacRae et al., Gene 71, 339-348, 1988; MacRae et al., Gene 132, 193-198, 1993), tpiA- (McKnight et al., Cell 46, 143-147, 1986; Upshall et al., Bio/Technology 5, 1301-1304, 1987), gpdA- (Punt et al., Gene 69, 49-57, 1988; Punt et al., J. Biotechnol. 17, 19-37, 1991) and the pkiA-promoter (de Graaff et al., Curr. Genet. 22, 21-27, 1992). Suitable promoter elements which could be used for expression in yeast are known in the art and are, e.g., the pho5-promoter (Vogel et al., Mol. Cell. Biol. 9, 2050-2057, 1989; Rudolf and Hinnen, Proc. Natl. Acad. Sci. USA 84, 1340-1344, 1987) or the gap-promoter for expression in *Saccharomyces cerevisiae*, and e.g. the aox1-promoter for *Pichia pastoris* (Koutz et al., Yeast 5, 167-177, 1989; Sreekrishna et al., J. Basic Microbiol. 28, 265-278, 1988), or the FMD promoter (Hollenberg et al., EPA No. 0299108) or MOX promoter (Ledeboer et al., Nucleic Acids Res. 13, 3063-3082, 1985) for *H. polymorpha*.

Suitable promoters and vectors for bacterial expression include, e.g., a synthetic promoter described by Giacomini et al. (Gene 144, 17-24, 1994). Appropriate teachings for expression of the claimed (mutant) mevalonate kinases in bacteria, either by appropriate

plasmids or through integration of mevalonate kinase-encoding DNA sequences into the chromosomal DNA, can be found in many places, e.g., US patent No. 6,322,995.

The invention further concerns a host cell comprising the vector or plasmid of the invention. Suitable host cells may be eukaryotic or prokaryotic cells. Examples of suitable host cells include but are not limited to bacterial cells, such as cells of streptococci, staphylococci, enterococci, *E. coli*, *Streptomyces*, cyanobacteria, *Bacillus subtilis*, and *Streptococcus pneumoniae*; fungal cells, such as cells of a yeast *Kluyveromyces*, *Saccharomyces*, a basidiomycete, *Candida albicans* and *Aspergillus*; insect cells such as cells of *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS, HeLa, 3T3, BHK, 293, CV-1; and plant cells, such as cells of a gymnosperm or angiosperm.

Accordingly, vectors comprising a polynucleotide of the present invention, preferably for the expression of said polynucleotides in bacterial, fungal, yeast or plant hosts, and such transformed bacteria or fungal, yeast or plant hosts are also an object of the present invention.

15 The invention further relates to a method for producing an isoprenoid compound comprising:

(a) culturing the host cell of the invention in a suitable medium under conditions that allow expression of the modified mevalonate kinase in the host cell; and

(b) optionally separating the isoprenoid compound from the medium.

20 Such a method can be used for the biotechnological production of any type of isoprenoid compound or isoprenoid-derived compound: e.g., carotenoids such as, but not limited to, phytoene, lycopene, α -, β - and γ -carotene, lutein, zeaxanthin, β -cryptoxanthin, adonixanthin, echinenone, canthaxanthin, astaxanthin and derivatives thereof (Misawa & Shimada, J. Biotechnol. 59, 169-181, 1998; Miura et al., Appl. Environ. Microbiol. 64, 1226-1229, 1998; Hirschberg, Curr. Opin. Biotechnol. 10, 186-191, 1999; Margalith, Appl. Microbiol. Biotechnol. 51, 431-438, 1999; Schmidt-Dannert, Curr. Opin. Biotechnol. 11, 255-261, 2000; Sandmann, Arch. Biochem. Biophys. 385, 4-12, 2001; Lee & Schmidt-Dannert, Appl. Microbiol. Biotechnol. 60, 1-11, 2002); quinones such as, but not limited to, ubiquinone (= coenzyme Q), menaquinone, plastoquinones and anthraquinones, preferably coenzyme Q6, coenzyme Q7, coenzyme Q8, coenzyme Q9, coenzyme Q10 or coenzyme Q11, most preferably coenzyme Q10 (Clarke, Protoplasma 213, 134-147, 2000; Han et al., Plant Cell Tissue Organ Culture 67, 201-220, 2001; Kawamukai, J. Biosci. Bioeng. 94, 511-517, 2002); rubber and rubber derivatives, preferably natural rubber (= *cis*-1,4-polyisoprene; Mooibroek & Cornish, Appl. Microbiol. Biotechnol. 53, 355-365, 2000); sterols and sterol derivatives such as, but not limited to, ergosterol, cholesterol,

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hydrocortisone (Ménard Szczebra et al., *Nature Biotechnol.* 21, 143-149, 2003), vitamin D, 25-hydroxy-vitamin D3, dietary phytosterols (Ling & Jones, *Life Sci.* 57, 195-206, 1995) and natural surfactants (Holmberg, *Curr. Opin. Colloid. Interface Sci.* 6, 148-159, 2001); and a large number of other isoprenoids such as, but not limited to, monoterpenes, diterpenes, sesquiterpenes and triterpenes, *e.g.*, taxol (Jennewein & Croteau, *Appl. Microbiol. Biotechnol.* 57, 13-19, 2001) and gibberellins (Bruckner & Blechschmidt, *Crit. Rev. Biotechnol.* 11, 163-192, 1991).

Suitable host cells are all types of organisms that are amenable to genetic modification such as, but not limited to, bacteria, yeasts, fungi, algae, plants or animal cells. Methods of genetic and metabolic engineering are known to the man skilled in the art (*e.g.*, Verpoorte et al., *Biotechnol. Lett.* 21, 467-479, 1999; Verpoorte et al., *Transgenic Res.* 9, 323-343, 2000; Barkovich & Liao, *Metab. Eng.* 3, 27-39, 2001). Similarly, (potentially) suitable purification methods for isoprenoids and isoprenoid-derived compounds and/or molecules are well known in the area of fine chemical biosynthesis and production.

It is understood that a method for biotechnological production of an isoprenoid or isoprenoid-derived compound and/or molecule according to the present invention is not limited to whole-cellular fermentation processes as described above, but may also use, *e.g.*, permeabilized host cells, crude cell extracts, cell extracts clarified from cell remnants by, *e.g.*, centrifugation or filtration, or even reconstituted reaction pathways with isolated enzymes. Also combinations of such processes are in the scope of the present invention. In the case of cell-free biosynthesis (such as with reconstituted reaction pathways), it is irrelevant whether the isolated enzymes have been prepared by and isolated from a host cell, by *in vitro* transcription/translation, or by still other means.

The invention further relates to a method for producing a modified mevalonate kinase of the invention comprising:

- (a) culturing a host cell of the invention under conditions that allow expression of the modified mevalonate kinase of the invention; and
- (b) recovering the modified mevalonate kinase from the cells or from the media.

The modified mevalonate kinases of the invention may be prepared from genetically engineered host cells comprising expression systems.

For recombinant production of the polypeptides of the invention, host cells can be genetically engineered to incorporate polynucleotides or vectors or plasmids of the invention. Introduction of a polynucleotide or vector into the host cell can be effected by methods described in many standard laboratory manuals [*e.g.*, Davis et al., *Basic Methods*

in Molecular Biology (1986), and Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. (1989)] such as calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, ballistic introduction and infection.

A great variety of expression systems can be used to produce the modified mevalonate kinases of the invention. Such vectors include, among others, those described supra. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression in this regard.

In recombinant expression systems in eukaryotes, for secretion of a translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment appropriate secretion signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

Polypeptides of the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, and hydroxyapatite chromatography. In one embodiment, high performance liquid chromatography is employed for purification. Well known techniques for protein refolding may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification. Methods of protein purification are described in, e.g., Deutscher, Protein Purification, Academic Press, New York, 1990; and Scopes, Protein Purification, Springer Verlag, Heidelberg, 1994.

Mevalonate kinases of the present invention can be also expressed in plants according to methods as described, e.g., by Pen et al. in Bio/Technology 11, 811-814, 1994 or in EP 449 375, preferably in seeds as described, e.g., in EP 449 376. Some suitable examples of promoters and terminators include those from nopaline synthase (nos), octopine synthase (ocs) and cauliflower mosaic virus (CaMV) genes. One type of efficient plant promoter that may be used is a high level plant promoter. Such promoters, in operable linkage with the genetic sequences of the present invention should be capable of promoting expression of the present gene product. High level plant promoters that may be used in this invention include the promoter of the small subunit (ss) of the ribulose-1,5-bisphosphate carboxylase, for example from soybean (Berry-Lowe et al., J. Mol. Appl. Genet. 1, 483-498, 1982), and the promoter of the chlorophyll a/b binding protein. These two promoters are known to be light-induced in plant cells (see, for example, Genetic Engineering of Plants,

an Agricultural Perspective, A. Cashmore, Plenum Press, NY (1983), pages 29-38; Coruzzi et al., J. Biol. Chem. 258,1399-1402, 1983; and Dunsmuir et al., J. Mol. Appl. Genet. 2, 285-300, 1983).

Where commercial production of the instant proteins is desired, a variety of culture methodologies may be applied. For example, large-scale production of a specific gene product, overexpressed from a recombinant microbial host may be produced by both batch or continuous culture methodologies. Batch and fed-batch culturing methods are common and well known in the art and examples may be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, Mass., or Deshpande, Appl. Biochem. Biotechnol. 36, 227-234, 1992. Methods of modulating nutrients and growth factors for continuous culture processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, supra.

Fermentation media must contain suitable carbon substrates. Suitable substrates may include but are not limited to monosaccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks. It is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and will only be limited by the choice of organism.

The invention further relates to a method for the preparation of a mevalonate kinase having reduced sensitivity to feedback inhibition, comprising the following steps:

- (a) providing a polynucleotide encoding a first mevalonate kinase which exhibits sensitivity to feedback inhibition;
- (b) introducing one or more mutations into the polynucleotide sequence such that the mutated polynucleotide sequence encodes a second mevalonate kinase which contains at least one amino acid mutation when compared to the first mevalonate kinase wherein the at least one amino acid mutation is at one or more amino acid positions selected from the group consisting of amino acid positions corresponding to positions 17, 47, 93, 94, 132, 167, 169, 204, and 266 of the amino acid sequence as shown in SEQ ID NO:1;
- (c) optionally inserting the mutated polynucleotide in a vector or plasmid;
- (d) introducing the polynucleotide or the vector or plasmid into a suitable host cell; and

- (e) culturing the host cell under conditions that allow expression of the modified mevalonate kinase.

The preferred embodiments of this method correspond to the preferred embodiments of the modified mevalonate kinase, the polynucleotides encoding them, the vectors and plasmids, the host cells, and the methods described herein. The first and second mevalonate kinase correspond to the non-modified and modified mevalonate kinase, respectively (see supra).

Another aspect of the invention is the use of a modified mevalonate kinase of the invention or a polynucleotide of the invention for the manufacture of a medicament for the treatment of a disorder associated with decreased activity of mevalonate kinase. Such disorders include but are not limited to mevalonic aciduria, hyperimmunoglobulinemia D and periodic fever syndrome. It is preferred that a modified mevalonate kinase of the invention is administered as a therapeutic enzyme. The mode of administration includes oral, parenteral, intraperitoneal and/or subcutaneous administration. The modified mevalonate kinases of the invention and salts thereof can be formulated as pharmaceutical compositions (e.g. granules, enzyme crystals, tablets, pills, capsules, injections, solutions, and the like) comprising at least one such enzyme alone or in admixture with pharmaceutically acceptable carriers, excipients and/or diluents. The pharmaceutical compositions can be formulated in accordance with a conventional method. Specific dose levels for any particular patient will be employed depending upon a variety of factors including the activity of specific compounds employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy.

The polynucleotides of the invention may be used in a gene therapy protocol.

- Yet another aspect of the invention is the use of a modified mevalonate kinase of the invention or a polynucleotide of the invention for determining the concentration of mevalonate in biological fluids. Non-limiting examples of biological fluids are blood, serum, plasma, cerebrospinal fluid, urine, tears, sweat, as well as any other intracellular, intercellular and/or extracellular fluids.
- It is an object of the present invention to provide a polynucleotide comprising a nucleic acid sequence coding for a modified mevalonate kinase as described above, a vector, preferably an expression vector, comprising such a polynucleotide, a host cell which has been transformed by such a polynucleotide or vector, a process for the preparation of a mevalonate kinase of the present invention wherein the host cell as described before is cultured under suitable culture conditions and the mevalonate kinase is isolated from such

host cell or the culture medium by methods known in the art, and a process for the biotechnological production of isoprenoid(s) based on a host cell which has been transformed by such a polynucleotide or vector, and/or which may have stably integrated such a polynucleotide into its chromosome(s).

- 5 It is also an object of the present invention to provide (i) a DNA sequence which codes for a mevalonate kinase carrying at least one of the specific mutations of the present invention and which hybridizes under standard conditions with any of the DNA sequences of the specific modified mevalonate kinases of the present invention, or (ii) a DNA sequence which codes for a mevalonate kinase carrying at least one of the specific mutations of the present invention but, because of the degeneracy of the genetic code, does not hybridize
10 but which codes for a polypeptide with exactly the same amino acid sequence as a DNA sequence which hybridizes under standard conditions with any of the DNA sequences of the specific modified mevalonate kinases of the present invention, or (iii) a DNA sequence which is a fragment of such DNA sequences which maintains the activity properties of the polypeptide of which it is a fragment.
15

"Standard conditions" for hybridization mean in the context the conditions which are generally used by a man skilled in the art to detect specific hybridization signals and which are described, e.g. by Sambrook et al., "Molecular Cloning", second edition, Cold Spring Harbor Laboratory Press 1989, New York, or preferably so-called stringent hybridization
20 and non-stringent washing conditions or more preferably so-called stringent hybridization and stringent washing conditions a man skilled in the art is familiar with and which are described, e.g., in Sambrook et al. (s.a.). A specific example of stringent hybridization conditions is overnight incubation (e.g., 15 hours) at 42°C in a solution comprising: 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate
25 (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml of denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at about 65°C.

It is furthermore an object of the present invention to provide a DNA sequence which can be obtained by the so-called polymerase chain reaction method ("PCR") by PCR primers
30 designed on the basis of the specifically described DNA sequences of the present invention. It is understood that the so obtained DNA sequences code for mevalonate kinases with at least the same mutation as the ones from which they are designed and show comparable activity properties.

The various embodiments of the invention described herein may be cross-combined.

Figure 1: Multiple sequence alignment calculated with the program ClustalW of mevalonate kinase sequences from mouse, rat, man, yeast, *Arabidopsis thaliana* (ARATH), *Schizosaccharomyces pombe* (SCHPO), *Pyrococcus abyssi* (PYRAB), *Pyrococcus horikoshii* (PYRHO), *Pyrococcus furiosus* (PYRFU), *Methanobacterium thermoautotrophicum* (METTH), *Archaeoglobus fulgidus* (ARCFU), *Methanococcus jannaschii* (METJA), *Aeropyrum pernix* (AERPE), and *Paracoccus zeaxanthinifaciens* (PARACOCBUS).
5 Numbering is according to the amino acid sequence of *Paracoccus zeaxanthinifaciens* mevalonate kinase.

Figure 2: Introduction of the K93E mevalonate kinase mutation into the mevalonate operon on a pBBR-K-based plasmid. See text for details.
10

The following non-limiting examples further illustrate the invention.

Example 1: Multiple sequence alignment

A multiple amino acid sequence alignment of different mevalonate kinases (see Fig. 1) can be calculated, e.g., with the program "PILEUP" (GCG Wisconsin Package, version 10.2, Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752, USA) using the following
15 parameters: gap creation penalty 12, gap extension penalty 4, and blosum62.cmp matrix (default parameters); or with the program ClustalW (Version 1.7, EMBL, Heidelberg, Germany) using BLOSUM exchange matrix. Such sequence alignments are routinely performed by the man skilled in the art (e.g., Cho et al., J. Biol. Chem. 276, 12573-12578,
20 2001).

Homologous mevalonate kinases in the context of the present invention may show sequence similarity with any of the mevalonate kinases shown in Fig. 1. Figure 1 gives an example of a multiple sequence alignment for the mevalonate kinase amino acid sequences of mouse, rat, man, *Arabidopsis thaliana* (ARATH), *Schizosaccharomyces pombe* (SCHPO),
25 yeast (YEAST), *Pyrococcus abyssi* (PYRAB), *Pyrococcus horikoshii* (PYRHO), *Pyrococcus furiosus* (PYRFU), *Methanobacterium thermoautotrophicum* (METTH), *Archaeoglobus fulgidus* (ARCFU), *Methanococcus jannaschii* (METJA), *Aeropyrum pernix* (AERPE), and *Paracoccus zeaxanthinifaciens* (PARACOCBUS) which latter sequence is also used as the reference for amino acid numbering to which the positions of the other sequences, e.g. the
30 ones named before, are referred to. Furthermore the modified rat mevalonate kinase with the E6V mutation means nothing else than the mevalonate kinase of the rat wherein at position 6 according to the assignment as defined above (which is in fact position 4 of the rat mevalonate kinase amino acid sequence), the naturally occurring Glu ("E" refers to the standard IUPAC one letter amino acid code) has been replaced by Val ("V"). All
35 mutants/variants of the present invention are designated in this way.

Example 2: Measurement of mevalonate kinase activity and of inhibition by feedback inhibitors

Enzymatic assays for measuring mevalonate kinase activity have been described, e.g., by Popják (Meth. Enzymol. 15, 393-, 1969), Gibson et al. (Enzyme 41, 47-55, 1989), Hinson et al. (J. Lipid Res. 38, 2216-2223, 1997), Schulte et al. (Anal. Biochem. 269, 245-254, 1999), or Cho et al. (J. Biol. Chem. 276, 12573-12578, 2001). For preparing mevalonate as substrate, 130 mg of DL-mevalonate lactone (FLUKA Chemie AG, Buchs, Switzerland) were dissolved in 5.5 ml of 0.2 M KOH and incubated for 15 min at 50°C. The solution was then adjusted to pH 7.0 by addition of 0.1 M HCl at room temperature (RT). Except if stated otherwise (see Example 3), the assay mixture consists of: 100 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM ATP, 2 mM $MgCl_2$, 1 mM mevalonate, 0.5 mM phosphoenolpyruvate (PEP), 0.32 mM NADH, 20 U/ml pyruvate kinase and 27 U/ml lactate dehydrogenase (Sigma-Aldrich, St. Louis, MO, USA). FPP, GGPP, IPP, DMAPP and GPP tested as inhibitors in the assay mixtures (at concentrations of 0-100 μ M) were all purchased from Sigma. Upon addition of purified (His₆-tagged) mevalonate kinase, enzymatic reaction reflected by consumption of NADH was followed by photometric measurement at 340 nm. One unit (1 U) of mevalonate kinase activity catalyzes the phosphorylation of 1 μ mol of mevalonate per min.

Example 3: Testing of the quality of the enzymatic assay

An optimal assay should fulfill a number of requirements, such as linearity with enzyme concentration and linearity with time. In addition, in the context of the present invention, the assay should allow to quantify inhibition of mevalonate kinase by feedback inhibitors. In the experiments of this Example, the following assay conditions were used: 100 mM KH_2PO_4 , pH 7.0, 0.125-4 mM ATP, 1.125-5 mM $MgCl_2$ (always 1 mM in excess of ATP!), 0.25-3 mM mevalonate, 0 or 46 μ M FPP, 0.16 mM NADH, 0.5 mM PEP, 20 U/ml pyruvate kinase, 27 U/ml lactate dehydrogenase, 30°C. Different amounts of purified His₆-tagged *Paracoccus zeaxanthinifaciens* mevalonate kinase were used.

The experiments of this example show that the mevalonate kinase activity assay, in fact, is linear with time and enzyme (mevalonate kinase) concentration, and that under the given conditions for *Paracoccus zeaxanthinifaciens*, MgATP and mevalonate concentrations of 1 mM each may be optimal to allow reliable measurement of feedback inhibition by FPP.

Example 4: Mutagenesis of *Paracoccus zeaxanthinifaciens* mevalonate kinase to obtain feedback-resistant mutants

The cDNA of mevalonate kinase from *Paracoccus zeaxanthinifaciens* R114 is amplified by PCR using a primer encoding an *Eco*RI restriction site along with a sequence of 6xHis as

well as a piece of the 5'-end sequence of mevalonate kinase without the ATG start codon, and a primer containing the 3'-end sequence of mevalonate kinase including the stop codon and a *Bam*HI restriction site. After purification by agarose gel electrophoresis, the PCR product is digested by *Eco*RI and *Bam*HI and ligated into pQE-80L (Qiagen, Hilden, Germany), which is digested with the same enzymes. pQE-80L contains a T5 promoter regulated by a *lac* operator element, which can be *cis*-inhibited by the *lac* repressor also encoded by pQE-80L. The plasmid is then transformed into *E. coli* DH5 α of Invitrogen (Carlsbad, CA, USA) according to the supplier's protocol. Upon addition of 100 μ M IPTG at an OD_{600nm} of 0.6 during exponential growth phase of *E. coli*, His₆-tagged mevalonate kinase is induced at 30°C for 4 h by shaking at 250 rpm. Purification of His₆-tagged mevalonate kinase and of His₆-tagged mevalonate kinase mutant enzymes is done with Ni-NTA chromatography using the QIAexpress system/reagents of Qiagen.

Mutagenesis of His₆-tagged mevalonate kinase is achieved by the so-called "two step PCR" using Turbo-Pfu DNA polymerase of Stratagene (La Jolla, CA, USA). The first PCR is performed with a primer containing the mutated codons (primer M) and the primer pQE-5' corresponding to a piece of sequence at the 5'-end of the multiple cloning sites (MCS) of pQE-80L. The template is pQE-80L-His-Mvk. The PCR product is purified by agarose gel electrophoresis and used as a primer for the second PCR reaction also containing the primer pQE-3' encompassing a piece of the 3'-end sequence of the MCS and the wild-type pQE-80L-His-Mvk as template. The PCR product is purified by agarose gel electrophoresis and digested by *Eco*RI and *Bam*HI, with which the His-Mvk is subcloned in pQE-80L. Finally, the digested fragment is purified by agarose electrophoresis and ligated into pQE-80L linearized by the same restriction enzymes.

Example 5: Feedback resistance of mutants of *Paracoccus zeaxanthinifaciens* mevalonate kinase

Mevalonate was prepared as described in Example 2. The assay mixture consists of: 100 mM K₂HPO₄/KH₂PO₄ (pH 7.0), 1 mM ATP, 1 mM mevalonate, 2 mM MgCl₂, 0.5 mM phosphoenolpyruvate (PEP), 0.32 mM NADH, 20 U/ml pyruvate kinase and 27 U/ml lactate dehydrogenase (Sigma-Aldrich, St. Louis, MO, USA). FPP, GGPP, IPP, DMAPP and GPP tested as inhibitors in the assay mixtures were all purchased from Sigma. 92 μ M FPP or 17.6 μ M GGPP were used for inhibition assays performed with the mevalonate kinase mutants. For the comparison of inhibition by FPP, GGPP, IPP, DMAPP and GPP, 138 μ M of these intermediates were added (Example 9). Upon addition of purified (His₆-tagged) mevalonate kinase, enzymatic reaction reflected by consumption of NADH was followed by photometric measurement at 340 nm.

Feedback resistance (%) is calculated as follows: if a and b are the measured mevalonate kinase activities of the wild-type enzyme in the absence and presence, respectively, of the feedback inhibitor (in this case, FPP), and if c and d are the measured mevalonate kinase activities of the mutant enzyme in the absence and presence, respectively, of the same feedback inhibitor, then % feedback resistance is:

$$\% \text{ resistance} = 100 \cdot ((d/c) - (b/a)) / (1 - (b/a))$$

Table 1. Impact of mutagenesis of *Paracoccus zeaxanthinifaciens* mevalonate kinase on the specific activity and the feedback resistance of the enzyme.

Mutant	Specific activity (% of wild-type)	Feedback resistance (%)
WT	100	0
I17T	95	46
G47D	121	32
K93E	109	33
V94I	96	22
P132A, P375R	158	35
R167W, K169Q	50	43
R204H	83	7
C266S	64	14
I17T, G47D	77	42
I17T, K93E	72	51
I17T, R167W, K169Q	37	71
I17T, P132A, P375R	62	56
K93E, P132A, P375R	111	57

WT represents the mevalonate kinase with SEQ ID No:15 (with His₆-tag).

- 10 That these mutations have an impact on feedback inhibition of mevalonate kinase is surprising. Previously, a conserved, hydrophobic stretch from residue 133 to residue 156 of human mevalonate kinase has been proposed to be a good candidate for isoprenoid binding (Riou et al., Gene 148, 293-297, 1994; Houten et al., Biochim. Biophys. Acta 1529,

19-32, 2000). However, none of the above mutations is located in the corresponding stretch of *Paracoccus zeaxanthinifaciens* mevalonate kinase (residues 137-160).

A considerable number of mutations have been proposed to decrease or even destroy mevalonate kinase activity and, thus, to cause the human diseases mevalonic aciduria and hyperimmunoglobulinemia D and periodic fever syndrome (e.g., K13X, H20P, H20N, L39P, W62X, S135L, A148T, Y149X, S150L, P165L, P167L, G202R, T209A, R215Q, T243I, L264F, L265P, I268T, S272F, R277C, N301T, G309S, V310M, G326R, A334T, V377I, and R388X; all in human mevalonate kinase; Houten et al., Eur. J. Hum. Genet. 9, 253-259, 2001; Cuisset et al., Eur. J. Hum. Genet. 9, 260-266, 2001). Of these, only two (i.e., P165L and R215Q) occur at residues corresponding in position within the amino acid sequence alignment with residues of *Paracoccus zeaxanthinifaciens* mevalonate kinase shown to have an impact on feedback resistance (i.e., residues 169 and 204, respectively). However, the previously described mutations in human mevalonate kinase were not shown to have an effect on feedback resistance, but were rather suggested to negatively impact the (specific) activity of the enzyme.

Example 6: Saturated mutagenesis of *Paracoccus zeaxanthinifaciens* mevalonate kinase at amino acid residues/positions previously identified to have an impact on the resistance of the enzyme to feedback inhibition

Saturated mutagenesis was done in the same way as described above for mutagenesis, except that the mutagenesis primer was synthesized in a way that the codons subject to saturated mutagenesis were made of randomized sequence.

Table 2. Saturated mutagenesis of residues 167 and 169 in the *Paracoccus zeaxanthinifaciens* mevalonate kinase mutant I17T, and impact on specific activity and feedback resistance of the enzyme.

Mutant	Specific activity (% of wild-type)	Feedback resistance (%)
WT	100	0
I17T, R167W, K169Q	37	71
I17T, R167W, K169H	43	67
I17T, R167T, K169M	54	57
I17T, R167T, K169Y	40	66
I17T, R167F, K169Q	43	77
I17T, R167I, K169N	35	73

I17T, R167H, K169Y	54	64
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WT represents the mevalonate kinase with SEQ ID NO:15 (with His₆-tag).

Table 3. Saturated mutagenesis of residue 93 in the *Paracoccus zeaxanthinifaciens* mevalonate kinase mutant I17T, G47D.

Mutant	Specific activity (% of wild-type)	Feedback resistance (%)
I17T, G47D, K93E	76	78
I17T, G47D, K93Q	83	76

5 Table 4. Saturated mutagenesis of residue 132 in the *Paracoccus zeaxanthinifaciens* mevalonate kinase mutant I17T, G47D, K93E.

Mutant	Specific activity (% of wild-type)	Feedback resistance (%)
I17T, G47D, K93E	76	78
I17T, G47D, K93E, P132A	90	79
I17T, G47D, K93E, P132S	100	83

Example 7: Improved production of the isoprenoid compound coenzyme Q10 using a feedback inhibition-resistant mevalonate kinase

- 10 To test the *in vivo* effect of mutations affecting feedback inhibition of mevalonate kinase, the *Paracoccus zeaxanthinifaciens* mevalonate kinase mutant K93E was introduced into a functional mevalonate operon cloned in a broad host range vector capable of replicating in *Paracoccus zeaxanthinifaciens*. The production of the isoprenoid compound coenzyme Q10 was compared directly in two recombinant strains of *P. zeaxanthinifaciens* that differ
- 15 only by the presence or absence of the K93E mutation.

Plasmid constructions

The plasmid constructions are depicted diagrammatically in Figure 2. The details of the cloning were as follows. *E. coli* strains were grown at 37°C in LB medium (Becton

Dickinson, Sparks, MD, USA). For maintenance of plasmids in recombinant *E. coli* strains, ampicillin (100 µg/ml) and/or kanamycin (25-50 µg/ml, depending on the experiment) were added to the culture medium. Agar (1.5% final concentration) was added for solid media. Liquid cultures were grown in a rotary shaker at 200 rpm.

- 5 Plasmid pBBR-K-mev-op-wt (Figure 2) contains the mevalonate operon, including its promoter region, from *P. zeaxanthinifaciens* strain ATCC 21588, inserted between the *SacI* and *NsiI* sites of plasmid pBBR1MCS-2 (Kovach et al., Gene 166, 175-176, 1995). The cloned mevalonate operon corresponds to the sequence from nucleotides 2469 to 9001 of the sequence having the GenBank/EMBL accession number AJ431696. Between the *SacI* site and the mevalonate operon sequence there is a short linker sequence, which is derived from plasmid pCR[®]2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and corresponds to the sequence from the *SacI* site to the PCR fragment insertion site. It should be noted that the sequence with accession number AJ431696 is from *P. zeaxanthinifaciens* strain R114 (ATCC PTA-3335), not from *P. zeaxanthinifaciens* strain ATCC 21588. The only difference between the mevalonate operon sequences of the *P. zeaxanthinifaciens* strains ATCC 21588 and R114 is a mutation in the *mvk* gene from strain R114. This mutation results in a change of amino acid 265 in the mevalonate kinase from alanine to valine (A265V). Because the mevalonate operon in pBBR-K-mev-op-wt is from ATCC 21588, it does not contain the mutation, thus codon 265 in *mvk* is GCC (and not GTC as in accession number AJ431696).

A plasmid analogous to pBBR-K-mev-op-wt but with the *mvk* gene from strain R114 was also constructed and was designated pBBR-K-mev-op-R114. Introduction of a *ddsA* gene from *P. zeaxanthinifaciens* strain ATCC 21588 under the control of the *crtE* promoter region between the *Ecl136 II* and the *SpeI* sites of pBBR-K-mev-op-R114 resulted in pBBR-K-mev-op-R114-P_{crtE}-*ddsA*_{wt} (Figure 2).

- The final step was to create a plasmid identical to pBBR-K-mev-op-R114-P_{crtE}-*ddsA*_{wt}, but containing the K93E mutation in the *mvk* gene. The plasmid pBlu2SP-*mvk-mvd* (Figure 2) was constructed by subcloning the 3166 bp *XmaI* - *SpeI* fragment in the *XmaI* - *SpeI* cut vector pBluescript II KS+ (Stratagene, La Jolla, CA, USA). Plasmid pBlu2SP-*mvk-mvd* has the convenient unique restriction endonuclease sites *XmaI* and *AscI* for the introduction of the mutated *mvk* gene into the 3' end of the mevalonate operon. Plasmid pQE-80L-*mvk*-K93E was cut with *XmaI* and *AscI* and the 1 kb fragment carrying most of *mvk*, including the K93E mutation, was ligated with the *XmaI* - *AscI* cut backbone of pBlu2SP-*mvk-mvd* resulting in pBlu2KSp-*mvk*-K93E-*mvd*. To reconstitute the full-length mevalonate operon with the K93E mutation in *mvk*, pBlu2KSp-*mvk*-K93E-*mvd* was cut with *XmaI* and *SpeI* and the 3166 bp fragment ligated with the 8.18 kb *XmaI* - *SpeI* fragment from pBBR-K-mev-op-R114-P_{crtE}-*ddsA*_{wt}, resulting in pBBR-K-mev-op-(*mvk*-K93E)-P_{crtE}-*ddsA*_{wt}. The

codon 265 of the *mvk* gene in this plasmid is GTC, because the *mvk* gene in pQE-80L-*mvk*-K93E is derived from *P. zeaxanthinifaciens* strain R114 (ATCC PTA-3335).

In summary, plasmids pBBR-K-mev-op-R114-*PcrtE-ddsA_{wt}* and pBBR-K-mev-op-(*mvk*-K93E)-*PcrtE-ddsA_{wt}* are identical except for the presence of the K93E mutation in the latter
5 plasmid.

Construction of recombinant *P. zeaxanthinifaciens* strains

P. zeaxanthinifaciens strains were grown at 28°C. The compositions of the media used for
P. zeaxanthinifaciens are described below. All liquid cultures of *P. zeaxanthinifaciens* grown
in flasks were shaken in a rotary shaker at 200 rpm unless specified otherwise. Agar (2%
10 final concentration) was added for solid medium. When media were sterilized by
autoclaving, the glucose was added (as a concentrated stock solution) after sterilization to
achieve the desired final concentration. F-Medium contains (per liter distilled water):
tryptone, 10 g; yeast extract, 10 g; NaCl, 30 g; D-glucose·H₂O, 10 g; MgSO₄·7H₂O, 5 g. The
pH is adjusted to 7.0 before sterilization by filtration or autoclaving. Medium 362F/2
15 contains (per liter distilled water): D-glucose·H₂O, 33 g; yeast extract, 10 g; tryptone, 10 g;
NaCl, 5 g; MgSO₄·7H₂O, 2.5 g. The pH of the medium is adjusted to 7.4 before
sterilization by filtration or autoclaving. Following sterilization, 2.5 ml each of
microelements solution, NKP solution and CaFe solution are added. The latter three
solutions are sterilized by filtration. Microelements solution contains (per liter distilled
20 water): (NH₄)₂Fe(SO₄)₂·6H₂O, 80 g; ZnSO₄·7H₂O, 6 g; MnSO₄·H₂O, 2 g; NiSO₄·6H₂O, 0.2
g; EDTA, 6 g. NKP solution contains (per liter distilled water): K₂HPO₄, 250 g; (NH₄)₂PO₄,
300 g. CaFe solution contains (per liter distilled water): CaCl₂·2H₂O, 75 g; FeCl₃·6H₂O, 5
g; concentrated HCl, 3.75 ml.

Preparation of electrocompetent cells of *P. zeaxanthinifaciens* strain R114 and
25 electroporation was performed as follows: 100 ml F medium was inoculated with 1.5 ml of
a stationary phase culture of *P. zeaxanthinifaciens* strain R114 and grown at 28°C, 200 rpm
until an optical density at 660 nm of about 0.5 was reached. The cells were harvested by
centrifugation for 15 minutes at 4°C, 7000 x g and washed twice in 100 ml ice-cold HEPES
buffer, pH 7. The final pellet was resuspended in 0.1 ml ice-cold HEPES buffer, pH 7 and
30 the cells were either used immediately for electroporation or glycerol was added to a final
concentration of 15% and the cells were stored in 50 µl aliquots at -80°C. One to five µl
plasmid DNA was added in salt-free solution and electroporations were performed at 18
kV/cm and 129 Ohms in ice-cooled 1-mm cuvettes. Pulse lengths were typically between 4
and 5 milliseconds. One ml of F medium was added and the cells were incubated for 1
35 hour at 28°C. Dilutions were spread onto F-agar plates containing 25-50 µg/ml kanamycin

and incubated at 28°C. Putative transformants were confirmed to contain the desired plasmid by PCR analysis.

Culture conditions for evaluating coenzyme Q10 production

Coenzyme Q10 production was tested in fed-batch cultivations of *P. zeaxanthinifaciens* strains R114/pBBR-K-mev-opR114-*PcrtE-ddsA_{wt}* and R114/pBBR-K-mev-op-(*mvk*-K93E)-*PcrtE-ddsA_{wt}*. All cultures were initiated from frozen cell suspensions (stored as 25% glycerol stocks at -80°C). The precultures for the fed-batch fermentations were prepared in duplicate 2-liter baffled shake flasks containing 200 ml of 362F/2 medium each. Two milliliters of thawed cell suspension were used as inoculum for each flask. The initial pH of the precultures was 7.2. The precultures were incubated at 28°C with shaking at 250 rpm for 28 hours, after which time the optical density at 660 nm (OD₆₆₀) was between 14 and 22 absorbance units, depending on the strain used. Main cultures were grown in Biostat ED Bioreactors (B. Braun Biotech International, Melsungen, Germany) containing medium having the following composition (per liter distilled water): D-glucose·H₂O, 25 g; yeast extract (Tastone 900), 17 g; NaCl, 4.0 g; MgSO₄·7H₂O, 6.25 g; (NH₄)₂Fe(SO₄)₂·6H₂O, 0.5 g; ZnSO₄·7H₂O, 0.038 g; MnSO₄·H₂O, 0.013 g; NiSO₄·6H₂O, 0.001 g; CaCl₂·2H₂O, 0.47 g; FeCl₃·6H₂O, 0.062 g; niacin, 0.01 g; NH₄Cl, 0.5 g; antifoam, 0.1 ml; KP solution, 3.5 ml. The composition of KP solution is (per liter distilled water): K₂HPO₄, 250 g; NaH₂PO₄·2H₂O, 200 g; (NH₄)₂HPO₄, 100 g. kanamycin (50 mg/l final concentration) was added to the medium for plasmid-carrying strains. The feeding solution used in all processes had the following composition (per liter distilled water): D-glucose·H₂O, 550 g; KP solution, 18.25 ml. The initial volume in the bioreactor (after inoculation) was 8.0 L. Precultures were diluted as needed with sterile water such that addition of 400 ml to the bioreactor achieved an initial OD₆₆₀ value of 0.5. Fermentation conditions were automatically controlled as follows: 28°C, pH 7.2 (pH controlled with addition of 28% NH₄OH), dissolved oxygen controlled at a minimum of 40% relative value (in cascade with agitation), minimum agitation of 300 rpm and an aeration rate of 1 v.v.m. (relative to final volume). The cultivations proceeded under these conditions without addition of feed solution for about 20 hours (batch phase). After this time, a decrease in agitation speed, cessation of base consumption, a sharp pH increase and a decrease in CO₂ production were the indication that the initial glucose was exhausted and the feeding was started. A standard feed profile was defined as follows (from feeding start point): ramp from 50 g/h to 80 g/h in 17 hours, continue at 80 g/h for 7 hours then ramp down to 55 g/h in 11 hours and continue at 55 g/h for the rest of the fermentation (total fermentation time = 70 hours). The final volumes of the main cultures were about 10 liters.

Analytical methods

Reagents. Acetonitrile, dimethylsulfoxide (DMSO), tetrahydrofuran (THF), tert-butyl methyl ether (TBME) and butylated hydroxytoluene (BHT) were puriss., p.a. or HPLC grade and were obtained from Fluka (Switzerland). Coenzyme Q10 was purchased from Fluka. Methanol (Lichrosolv) was purchased from Merck, Darmstadt, Germany.

5 Carotenoid standards were obtained from the Chemistry Research Department, Roche Vitamins Ltd., Switzerland.

Sample preparation and extraction. Four hundred microliters of whole broth were transferred to a disposable 15 ml polypropylene centrifuge tube. Four milliliters of stabilized extraction solution (0.5 g/l BHT in 1:1 (v/v) DMSO/THF) were added and the

10 samples were mixed for 20 minutes in a laboratory shaker (IKA, Germany) to enhance extraction. Finally, the samples were centrifuged and the supernatants were transferred to amber glass vials for analysis by high performance liquid chromatography (HPLC).

HPLC. A reversed phase HPLC method was developed for the simultaneous determination of ubiquinones and their corresponding hydroquinones. The method is able to clearly

15 separate the carotenoids zeaxanthin, phytoene, β -cryptoxanthin, β -carotene and lycopene from coenzyme Q10. Chromatography was performed using an Agilent 1100 HPLC system equipped with a temperature-controlled autosampler and a diode array detector. The method parameters were as follows:

20	Column	YMC Carotenoid C30 column 3 micron, steel, 150 mm length x 3.0 mm I.D. (YMC, Part No. CT99S031503QT)			
	Guard column	Security Guard C18 (ODS, Octadecyl) 4 mm length x 3.0 mm I.D. (Phenomenex, Part No. AJO-4287)			
25	Typical column pressure	60 bar at start			
	Flow rate	0.5 ml/min			
	Mobile phase	Mixture of acetonitrile(A):methanol(B):TBME(C)			
30	Gradient profile	<u>Time (min)</u>	<u>%A</u>	<u>%B</u>	<u>%C</u>
		0	60	15	25
		13	60	15	25
		20	0	0	100
		22	60	15	25
		22	60	15	25

Post time 4 minutes

Injection volume 10 μ l

Column temperature 15°C

Detection Three wavelengths were used for detection of specific compounds according to Table 5.

5

Table 5. HPLC retention times and wavelengths used.

Compound	Wavelength (nm)	Retention times (min)
Zeaxanthin (Z-isomers)	450	4.2, 6.4
E-Zeaxanthin	450	5.2
Phytoene	280	7.7
β -Cryptoxanthin	450	8.6
Ubiquinol 10	210	11.4
Coenzyme Q10	210	12.8
β -Carotene	450	14.5
Lycopene	450	22.0

Calculations, selectivity, linearity, limit of detection and reproducibility. Calculations were based on peak areas. The selectivity of the method was verified by injecting standard solutions of the relevant reference compounds. The target compounds (coenzyme Q10 and ubiquinol 10) were completely separated and showed no interference. A dilution series of coenzyme Q10 in extraction solution (see above) was prepared and analyzed. A linear range was found from 5 mg/l to 50 mg/l. The correlation coefficient was 0.9999.

The limit of detection for coenzyme Q10 by this HPLC method was determined to be 4 mg/l. The reproducibility of the method including the extraction procedure was checked. Ten individual sample preparations were compared. The relative standard deviation was determined to be 4%.

Coenzyme Q10 production results

Under the fed-batch cultivation conditions described above, the final concentration of coenzyme Q10 produced by *P. zeaxanthinifaciens* strain R114/pBBR-K-mev-op-(*mvk*-K93E)-*PcrtE-ddsA_{wt}* was 34% higher than observed for strain R114/pBBR-K-mev-opR114-*PcrtE-ddsA_{wt}*. This difference was not attributable simply to differences in the growth of the two strains, as strain R114/pBBR-K-mev-op-(*mvk*-K93E)-*PcrtE-ddsA_{wt}* also showed a 12% higher specific coenzyme Q10 production (units coenzyme Q10/gram cell dry mass/hour) compared to strain R114/pBBR-K-mev-opR114-*PcrtE-ddsA_{wt}*. Further, strain R114/pBBR-K-mev-op-(*mvk*-K93E)-*PcrtE-ddsA_{wt}* also showed a 31% decrease on mevalonate accumulation in the broth compared to strain R114/pBBR-K-mev-opR114-*PcrtE-ddsA_{wt}*. This comparison showed that the K93E mutation in plasmid pBBR-K-mev-op-(*mvk*-K93E)-*PcrtE-ddsA_{wt}* is directly responsible for the improved production of coenzyme Q10.

Example 8: Effect of the I17T mutation on the solubility of *Paracoccus zeaxanthinifaciens* mevalonate kinase

For human mevalonate kinase, mutants E19A, E19Q and H20A were shown to be completely insoluble after IPTG-induction of *E. coli* transformants (Potter and Mizioro, J. Biol. Chem. 272, 25449-25454, 1997). The His₆-tagged *Paracoccus zeaxanthinifaciens* R114 mevalonate kinase (SEQ ID NO:15) also displayed a pronounced tendency to aggregate/precipitate, in particular in buffer solutions with rather high ionic strength (e.g., 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole). Surprisingly, the His₆-tagged *Paracoccus zeaxanthinifaciens* R114 mevalonate kinase mutant I17T was completely soluble and stable under the same conditions, so that this mutant enzyme is much better suited for applications requiring soluble mevalonate kinase.

Example 9: Feedback inhibition of mevalonate kinase with different downstream products of the pathway

Different mevalonate kinases were previously reported to be sensitive to feedback inhibition by the following downstream products of the mevalonate pathway: IPP, DMAPP, GPP, FPP, GGPP, phytyl-PP, farnesol, dolichol phosphate. At 138 µM of GGPP, FPP, GPP, IPP, or DMAPP, the activity of His₆-tagged *Paracoccus zeaxanthinifaciens* mevalonate kinase was inhibited by 98%, 80.1%, 18.6%, 16.3% and 14.7%, respectively. The resistance of the *Paracoccus zeaxanthinifaciens* mevalonate kinase mutant I17T/G47D/K93E/P132S to feedback inhibition by FPP (92 µM) or GGPP (17.6 µM) was 83% and 92%, respectively.

Example 10: Identification of corresponding residues in mevalonate kinases that are homologous to *Paracoccus zeaxanthinifaciens* mevalonate kinase

With the sequence alignment program GAP (GCG Wisconsin Package, version 10.2, Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752, USA; gap creation penalty 8; gap extension penalty 2), the following residues corresponding to specific amino acid positions of the amino acid sequence of *Paracoccus zeaxanthinifaciens* mevalonate kinase (SEQ ID NO:1) were identified:

SEQ ID NO:	Amino acid position								
1	I17	G74	K93	V94	P132	R167	K169	R204	C266
2	I15	S45	K90	V94	(-)	E163	P165	R215	C275
3	I15	S45	P83	T84	P127	P167	K169	R215	C275
4	I15	S45	K93	V94	L129	R171	S173	R215	C275
5	I12	P43	S83	T84	P131	E167	E179	K215	D269
6	I14	S45	Q93	E94	N131	L172	K174	K216	C279
7	I14	N44	V76	Q77	P120	P162	S164	R208	I268
8	I14	G46	E80	V81	(-)	L136	L138	Y173	S238
9	I14	G46	E80	V81	(-)	L136	L138	Y173	S238
10	I12	(-)	K78	A79	(-)	L135	L137	F172	V227
11	I12	T37	(-)	(-)	P80	R115	H117	Y152	I208
12	I10	S35	(-)	(-)	G76	G111	M113	(-)	D197
13	I10	Q40	(-)	(-)	T93	K129	L131	E166	I220
14	I14	(-)	S58	A59	P93	D128	L130	A165	I223
15	I26	G56	K102	V103	P141	R176	K178	R213	C275
30	I13	(-)	S86	I87	P135	R178	T184	K224	C290

Amino acid numbering according to the respective sequences SEQ ID NOs:1-15 and 30. (-) No homologous residue has been identified.

Examples of amino acid sequences of non-modified mevalonate kinases include but are not limited to the following amino acid sequences (SEQ ID NOs: 1-15 and 30). The nucleotide sequences encoding the non-modified mevalonate kinases (SEQ ID NOs:1-14 and 30) are shown in SEQ ID NOs:16-29 and 31, respectively.

SEQ ID No:1: Amino acid sequence of *Paracoccus zeaxanthinifaciens* mevalonate kinase.

- SEQ ID NO:2: Amino acid sequence of human mevalonate kinase (Swiss-Prot accession no. Q03426).
- SEQ ID NO:3: Amino acid sequence of mouse mevalonate kinase (Swiss-Prot accession no. Q9R008).
- 5 SEQ ID NO:4: Amino acid sequence of rat mevalonate kinase (Swiss-Prot accession no. P17256).
- SEQ ID NO:5: Amino acid sequence of *Arabidopsis thaliana* mevalonate kinase (Swiss-Prot accession no. P46086).
- 10 SEQ ID NO:6: Amino acid sequence of yeast mevalonate kinase (Swiss-Prot accession no. P07277).
- SEQ ID NO:7: Amino acid sequence of *Schizosaccharomyces pombe* mevalonate kinase (Swiss-Prot accession no. Q09780).
- SEQ ID NO:8: Amino acid sequence of *Pyrococcus abyssi* mevalonate kinase (Swiss-Prot accession no. Q9V187).
- 15 SEQ ID NO:9: Amino acid sequence of *Pyrococcus horikoshii* mevalonate kinase (Swiss-Prot accession no. O59291).
- SEQ ID NO:10: Amino acid sequence of *Pyrococcus furiosus* mevalonate kinase (Swiss-Prot accession no. Q8U0F3).
- 20 SEQ ID NO:11: Amino acid sequence of *Methanobacterium thermoautotrophicum* mevalonate kinase (Swiss-Prot accession no. Q50559).
- SEQ ID NO:12: Amino acid sequence of *Archaeoglobus fulgidus* mevalonate kinase (Swiss-Prot accession no. O27995).
- SEQ ID NO:13: Amino acid sequence of *Methanococcus jannaschii* mevalonate kinase (Swiss-Prot accession no. Q58487).
- 25 SEQ ID NO:13: Amino acid sequence of *Methanococcus jannaschii* mevalonate kinase (Swiss-Prot accession no. Q58487).
- SEQ ID NO:14: Amino acid sequence of *Aeropyrum pernix* mevalonate kinase (Swiss-Prot accession no. Q9Y946).

SEQ ID NO:15: Amino acid sequence of His₆-tagged mevalonate kinase of *Paracoccus zeaxanthinifaciens*.

SEQ ID NO:16: DNA sequence of *Paracoccus zeaxanthinifaciens* mevalonate kinase.

5 SEQ ID NO:17: DNA sequence of human mevalonate kinase (Genbank accession no. M88468).

SEQ ID NO:18: DNA sequence of mouse mevalonate kinase (Genbank accession no. AF137598).

SEQ ID NO:19: DNA sequence of rat mevalonate kinase (Genbank accession no. M29472).

10 SEQ ID NO:20: DNA sequence of *Arabidopsis thaliana* mevalonate kinase (Genbank accession no. X77793).

SEQ ID NO:21: DNA sequence of yeast mevalonate kinase (Genbank accession no. X06114).

SEQ ID NO:22: DNA sequence of *Schizosaccharomyces pombe* mevalonate kinase (Genbank accession no. AB000541).

15 SEQ ID NO:23: DNA sequence of *Pyrococcus abyssi* mevalonate kinase (Genbank accession no. AJ248284).

SEQ ID NO:24: DNA sequence of *Pyrococcus horikoshii* mevalonate kinase (Genbank accession no. AB009515; reverse direction).

20 SEQ ID NO:25: DNA sequence of *Pyrococcus furiosus* mevalonate kinase (Genbank accession no. AE010263; reverse direction).

SEQ ID NO:26: DNA sequence of *Methanobacterium thermoautotrophicum* mevalonate kinase (Genbank accession no. U47134).

SEQ ID NO:27: DNA sequence of *Archaeoglobus fulgidus* mevalonate kinase (Genbank accession no. AE000946; reverse direction).

25 SEQ ID NO:28: DNA sequence of *Methanococcus jannaschii* mevalonate kinase (Genbank accession no. U67551).

SEQ ID NO:29: DNA sequence of *Aeropyrum pernix* mevalonate kinase (Genbank accession no. AP000064).

SEQ ID NO:30: Amino acid sequence of *Phaffia rhodozyma* ATCC96594 mevalonate kinase.

SEQ ID NO:31: Gene (DNA) sequence of *Phaffia rhodozyma* ATCC96594 mevalonate kinase. The mevalonate kinase gene consists of 4 introns and 5 exons.

5	Exon 1:	1021-1124
	Intron 1:	1125-1630
	Exon 2:	1631-1956
	Intron 2:	1957-2051
	Exon 3:	2052-2366
10	Intron 3:	2367-2446
	Exon 4:	2447-2651
	Intron 4:	2652-2732
	Exon 5:	2733-3188
	PolyA site:	3284

15 SEQ ID NO:32: DNA sequence of the His₆-tagged *Paracoccus zeaxanthinifaciens* mevalonate kinase mutant I17T.

SEQ ID NO:33: DNA sequence of the His₆-tagged *Paracoccus zeaxanthinifaciens* mevalonate kinase mutant I17T/G47D/K93E/P132S.